Renal dysfunction induced by kidney-specific gene deletion of Hsd11b2 as a primary cause of salt-dependent hypertension

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Abstract: Genome-wide analysis of renal sodium-transporting system has identified specific variations of Mendelian hypertensive disorders, including HSD11B2 gene variants in apparent mineralocorticoid excess. However, these genetic variations in extrarenal tissue can be involved in developing hypertension, as demonstrated in former studies using global and brain-specific Hsd11b2 knockout rodents. To re-examine the importance of renal dysfunction on developing hypertension, we generated kidney-specific Hsd11b2 knockout mice. The knockout mice exhibited systemic hypertension, which was abolished by reducing salt intake, suggesting its salt-dependency. In addition, we detected an increase in renal membrane expressions of cleaved epithelial sodium channel- and T53-phosphorylated Na+-Cl− cotransporter in the knockout mice. Acute intraperitoneal administration of amiloride-induced natriuresis and increased urinary sodium/potassium ratio more in the knockout mice compared with those in the wild-type control mice. Chronic administration of amiloride and high-KCl diet significantly decreased mean blood pressure in the knockout mice, which was accompanied with the correction of hypokalemia and the resultant decrease in Na+-Cl− cotransporter phosphorylation. Accordingly, a Na+-Cl− cotransporter blocker hydrochlorothiazide significantly decreased mean blood pressure in the knockout mice. Chronic administration of mineralocorticoid receptor antagonist spironolactone significantly decreased mean blood pressure of the knockout mice along with downregulation of cleaved epithelial sodium channel- and phosphorylated Na+-Cl− cotransporter expression in the knockout kidney. Our data suggest that kidney-specific deficiency of 11 -HSD2 leads to salt-dependent hypertension, which is attributed to mineralocorticoid receptor–epithelial sodium channel–Na+-Cl− cotransporter activation in the kidney, and provides evidence that renal dysfunction is essential for developing the phenotype of apparent mineralocorticoid excess.

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Original Article

Renal Dysfunction Induced by Kidney-Specific Gene Deletion of *Hsd11b2* as a Primary Cause of Salt-Dependent Hypertension

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Abstract—Genome-wide analysis of renal sodium-transporting system has identified specific variations of Mendelian hypertensive disorders, including *HSD11B2* gene variants in apparent mineralocorticoid excess. However, these genetic variations in extrarenal tissue can be involved in developing hypertension, as demonstrated in former studies using global and brain-specific *Hsd11b2* knockout rodents. To re-examine the importance of renal dysfunction on developing hypertension, we generated kidney-specific *Hsd11b2* knockout mice. The knockout mice exhibited systemic hypertension, which was abolished by reducing salt intake, suggesting its salt-dependency. In addition, we detected an increase in renal membrane expressions of cleaved epithelial sodium channel-α and T53-phosphorylated Na^+-Cl^- cotransporter in the knockout mice. Acute intraperitoneal administration of amiloride-induced natriuresis and increased urinary sodium/potassium ratio more in the knockout mice compared with those in the wild-type control mice. Chronic administration of amiloride and high-KCl diet significantly decreased mean blood pressure in the knockout mice, which was accompanied with the correction of hypokalemia and the resultant decrease in Na^+-Cl^- cotransporter phosphorylation. Accordingly, a Na^+-Cl^- cotransporter blocker hydrochlorothiazide significantly decreased mean blood pressure in the knockout mice. Chronic administration of mineralocorticoid receptor antagonist spironolactone significantly decreased mean blood pressure of the knockout mice along with downregulation of cleaved epithelial sodium channel-α and phosphorylated Na^+-Cl^- cotransporter expression in the knockout kidney. Our data suggest that kidney-specific deficiency of 11β-HSD2 leads to salt-dependent hypertension, which is attributed to mineralocorticoid receptor–epithelial sodium channel–Na^+-Cl^- cotransporter activation in the kidney, and provides evidence that renal dysfunction is essential for developing the phenotype of apparent mineralocorticoid excess. (***Hypertension***. **2017;70:00-00. DOI: 10.1161/HYPERTENSIONAHA.116.08966.)** ● **Online Data Supplement**

Key Words: apparent mineralocorticoid excess syndrome ■ blood pressure ■ hypertension ■ hypokalemia ■ mineralocorticoid receptor

In the 1960s, the basic concept of blood pressure (BP) response to salt-intake was proposed.[1] In the direct measurement of the hemodynamics of partially-nephrectomized dogs, it was demonstrated that the daily infusion of isotonic saline led to an increase in the cardiac output and BP within a few days, which was associated with a slight decrease in the total peripheral vascular resistance. The results of numerous animal and human studies[2,3] confirmed Guyton proposal that the renal dysfunction primarily induces hypertension, and this has been accepted as the basic mechanism of salt-sensitive hypertension.

Almost all genetic variants identified in patients with Mendelian hypertensive disorders were found at the genetic loci associated with renal sodium-transporting system, such as *WNK1/4* gene in Gordon syndrome,[4] *SCNN1B/G* in Liddle syndrome,[5,6] and *HSD11B2* in the apparent mineralocorticoid excess (AME) syndrome.[7] These reports further supported Guyton proposal, even though Mendelian hypertensive disorders account for <1% of all human hypertension cases.

However, some scientists have recently expressed their criticism of Guyton proposal, questioning the primary role of renal dysfunction in the development of hypertension, suggesting that the observed genetic variations in patients with Mendelian hypertensive disorders may play a role in extrarenal organs as well, such as vasculature and brain.[8]

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These genes were, indeed, shown to be expressed in both kidneys and other tissues.[9–11] Therefore, it is necessary to examine whether an impaired regulation of the renal sodium transport represents a primary cause of salt-sensitive hypertension.

To elucidate this, we focused on the loss-of-function mutations of *HSD11B2* gene, found in patients with AME syndrome. 11β-Hydroxysteroid dehydrogenase (11β-HSD2 and HSD11B2) converts glucocorticoids to their inactive form and maintains the sensitivity of mineralocorticoid receptor (MR) to mineralocorticoids because glucocorticoids can bind to MR, and they are found in plasma at the concentrations 100× to 1000× higher than mineralocorticoids.[12,13] Therefore, the development of hypertension in patients with AME syndrome may potentially be ascribed to the aberrant activation of MR and epithelial sodium channel (ENaC) in the kidney. However, previous studies using *Hsd11b2* knockout mice reported that the natriuretic response to ENaC blockers did not differ between the knockout mice and the wild-type (WT) controls, which casts some doubt on the renal origin of salt-sensitive hypertension in this AME animal model.[14] Furthermore, brain-specific *Hsd11b2* knockout mice were shown to have a normal BP when fed with normal salt diet but extremely high-salt diet led to hypertension with impaired baroreflex,[15] which was associated with the hypertension ascribed to the increased production of catecholamines in the global knockout mice on normal salt diet.[16] These results support the "neuro-vascular" hypothesis, but plasma levels of catecholamines were normal in a patient with AME syndrome.[17] It is, therefore, still unknown how HSD11B2 deficiency leads to hypertension without high-salt intake, as observed in the global *Hsd11b2* knockout mice and in patients with AME syndrome.

To examine whether renal dysfunction plays a decisive role in the development of hypertension in patients with AME syndrome, we generated kidney-specific *Hsd11b2* knockout (*Hsd11b2*$^{Ksp-/-}$; knockout) mice by using kidney-specific cadherin (Ksp) promoter-controlled Cre/LoxP system.[18] We demonstrate that *Hsd11b2*$^{Ksp-/-}$ mice developed hypertension even with normal salt intake through the activation of renal MR–ENaC, which lead to hypokalemia and Na^+-Cl^- cotransporter (NCC) phosphorylation. Our data elucidate the underlying mechanisms of hypertension and the importance of renal dysfunction in developing AME syndrome.

Methods

Precise methods are available only in the online-only Data Supplement.

Animals

Animal care and treatment complied with the standards described in the Guidelines for the Care and Use of Laboratory Animals of the University of Tokyo, Japan. We used 8- to 20-week-old male C57BL/6-background *Hsd11b2*$^{Ksp-/-}$ mice and the age-matched Cre-negative male mice as WT control. All animals had free access to drinking water and diet and were grown under the temperature-controlled conditions, with 12 hours/12 hours of light (8:00 AM to 8:00 PM)/dark (8:00 PM to 8:00 AM). Normal-salt diet contained ≈0.35% Na^+, whereas low-salt diet contained 0.01% Na^+. High-potassium diet contained 8% potassium chloride (KCl). Amiloride (25 mg/L) and hydrochlorothiazide (300 mg/L) were administered through the drinking water. Slow-release pellets containing spironolactone (mineralocorticoid receptor antagonist, MRA; Innovative Research of America, Sarasota, FL) were produced to be capable for releasing the drug continuously for 21 days at the dose of 50 mg/kg body weight/d. The pellet was subcutaneously implanted into the neck. Chronic treatments of amiloride, 8%KCl diet, hydrochlorothiazide, and MRA were continued for 10 days. Before the surgery or euthanization, the animals were inhalationally anesthetized with 1.5% isoflurane.

BP Measurement

Arterial BP in conscious mice was directly monitored by catheterization of left carotid artery with radiotelemetry.

Antibodies

Primary antibodies used in this study were as follows: anti-ENaCα[19] (1/5000 for Western blotting [WB]), anti-NCC (1/5000 for WB, AB3553; Millipore), anti-pNCC T53[19] (1/5000 for WB), and anti-NKCC2 (1/5000 for WB, AB3562P; Abcam).

Statistical Analysis

The data are presented as mean± SEM. Comparisons between 2 groups were performed by using the Welch *t* test. Multiple comparisons were performed by using ANOVA with post hoc Bonferroni–Holm test. $P<0.05$ was considered statistically significant.

Results

Kidney-Specific Gene Deletion of *Hsd11b2*

Initially, we confirmed kidney-specific deletion of *Hsd11b2* gene in *Hsd11b2*$^{Ksp-/-}$ mice. Western blotting and real-time polymerase chain reaction analyses revealed that HSD11B2 expression is significantly decreased in the knockout mouse kidneys compared with that in the WT mouse kidneys, although colon expression did not differ between these mice (Figure S1A and S1B). Renal expression of HSD11B1 did not change (Figure S2). Double AQP2 and HSD11B2 staining showed that HSD11B2 colocalized in renal principal cells of the WT mouse kidneys, whereas the knockout mouse kidneys completely lacked the expression of HSD11B2 (Figure S1C). We also checked whether gene expressions were altered in aorta and brain stem of the knockout mice, which might regulate BP through modulating vascular resistance or sympathetic nervous system (SNS) activity. As expected, *Hsd11b2* gene expression was significantly decreased only in the knockout kidney and *Hsd11b1* gene expression was not altered between the genotypes in all of these tissues (Figure S3). The ratio of 11-dehydrocorticosterone to corticosterone in urine samples was significantly decreased in the knockout mice (Figure S4A).

Hypertension Is Salt-Dependent in Kidney-Specific Knockout Mice

We evaluated whether kidney-specific deletion of *Hsd11b2* gene affects BP and measured significantly higher systolic, diastolic, and mean arterial BP (MBP) values in the knockout mice than those determined in the WT mice by radiotelemetry (Figure 1A, 1B, and 1D), while both groups of mice were fed with normal salt diet and their food intake was at almost the same level (Figure S4B and S4C). Heart rate was significantly lower in the knockout mice than in the WT mice (Figure 1C). Although the elevation in systolic BP of the knockout mice was also confirmed by tail-cuff method (Figure S5A),

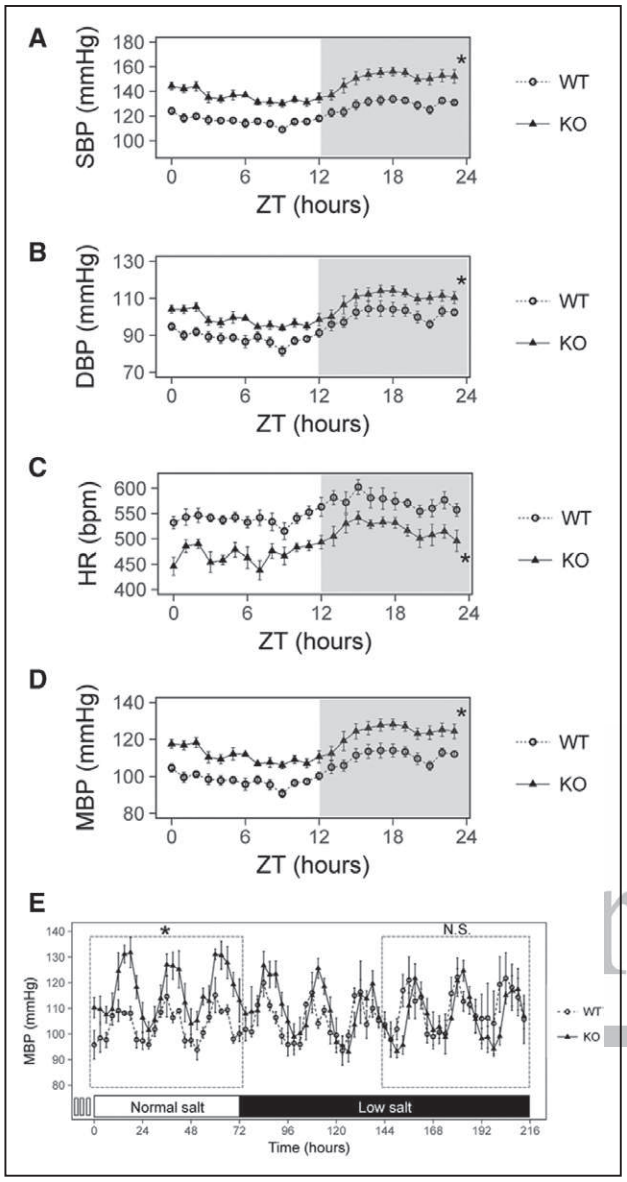

Figure 1. Salt-dependent blood pressure elevation in the kidney-specific *Hsd11b2* knockout (KO) mice. Systolic (SBP, **A**) and diastolic (DBP, **B**) arterial blood pressures and heart rate (HR, **C**) were measured for 3 d and averaged in *Hsd11b2*$^{KSP-/-}$ mice (n=7; filled triangles and solid line, KO) and flox-controlled littermates (n=7; open circles and dotted line, WT). Mean arterial blood pressure (MBP, **D**) was calculated from the obtained SBP and DBP values. ZT, zeitgeber time: ZT 0 to 12 is a light phase (white) and ZT 12 to 24 is a dark phase (gray) in a day. **E**, Averaged MBP values of *Hsd11b2*$^{KSP-/-}$ mice (n=4; filled triangles and solid line, KO) and flox-controlled littermates (n=4; open circles and dotted line, WT) in every 3 h are presented. MBP values of each group under normal salt diet (left dotted box) and low salt diet (right dotted box) were shown as mean±SEM and statistically analyzed by using 2-way ANOVA with post hoc Bonferroni–Holm test to assess the effects of the variables; genotype and time (**A** through **D**) or diet (**E**). *P<0.05, compared with the control in the same period.

Ksp-Cre transgene did not affect systolic BP in mice harboring WT *Hsd11b2* gene (Figure S5B). As expected, reducing dietary salt intake led to the normalization of the MBP in the knockout mice, reducing it to the levels determined in the WT mice (Figure 1E).

Hypokalemia and Suppression of the RAAS

Furthermore, both plasma aldosterone concentration and renin activity were significantly lower in the knockout mice than in the WT mice (Figure 2A and 2B), whereas corticosterone levels were not altered (Figure 2C). Hypokalemia represents another major symptom of AME syndrome. Here, we showed that plasma potassium levels were significantly reduced in the knockout mice compared with those in the WT mice (Figure 2D). Previously, hypokalemia observed in patients with AME syndrome has been described as a result of ENaC activation in the kidney, leading to the lowering of the urinary excretion of sodium and the increased excretion of potassium.

Acute Diuretic Response by Amiloride

Although it was previously reported that the increase in urinary sodium excretion induced by the ENaC blocker did not differ between global *Hsd11b2* knockout mice and their WT controls,[14] in our study, the acute natriuretic effect of ENaC blocker amiloride was shown to be significantly increased in the knockout mice, compared with that in the WT mice. Fractional excretion rate of sodium was significantly increased after the acute administration of amiloride in the knockout mice compared with that in the WT mice (Figure 3A). In addition, a decreased sodium/potassium excretion ratio was determined in the knockout mice treated

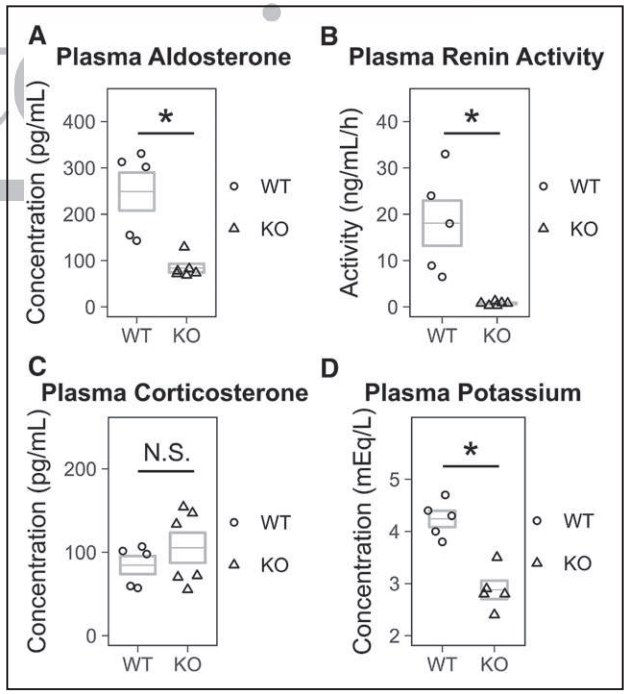

Figure 2. Apparent mineralocorticoid excess-like phenotypes observed in the kidney-specific *Hsd11b2* knockout (KO) mice. Blood samples were collected at the euthanization of *Hsd11b2*$^{KSP-/-}$ mice and flox-controlled littermates at around 8:00 PM. Plasma aldosterone levels (**A**), renin activity (**B**), and corticosterone levels (**C**) were determined (WT, wild-type [n=5]; KO [n=6]). Plasma potassium concentrations (**D**) were determined immediately after the euthanization using i-STAT (n=5 for each genotype). All data are shown as mean±SEM and statistically analyzed by using Welch *t* test. *P<0.05, compared with the control. N.S. indicates not significant.

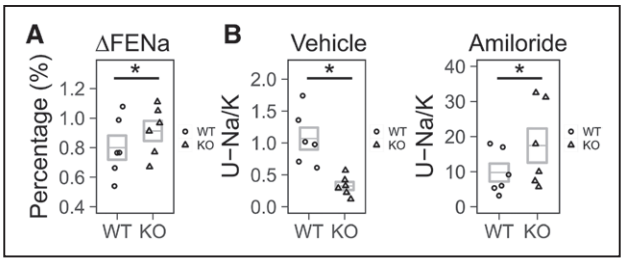

Figure 3. Acute amiloride test results of kidney-specific *Hsd11b2* knockout (KO) mice. Change of urinary fractional excretion ratio of sodium (ΔFENa; **A**) and urinary Na/K ratio in mice treated with vehicle and in those treated with amiloride (**B**) were analyzed using urine and blood samples (n=6 for each genotype). Data are shown as mean±SEM and statistically analyzed by using Welch *t* test. **P*<0.05, compared with the control. WT indicates wild-type.

with vehicle, in comparison with that in the vehicle-treated WT mice (Figure 3B) and, higher sodium/potassium excretion ratio in the amiloride-treated knockout mice was measured, compared with that in the amiloride-treated WT mice (Figure 3C).

Renal Membrane Expressions of Sodium Transporters

Furthermore, we evaluated the effect of kidney-specific HSD11B2 deficiency on the expression levels of sodium transporter proteins on membrane fractions (Figure S6) in the distal nephron, to determine the precise mechanism of aberrant sodium reabsorption in the knockout kidney. Western blot analyses of renal membrane fractions revealed an increase in the expressions of ENaCα and its cleaved form (Figure 4A and 4B), which were reported to be positive markers of ENaC activity.[19] NCC and T53-phosphorylated NCC19 levels were shown to be increased in the membrane fractions of the knockout mouse kidneys, while those of the sodium–potassium–chloride transporter (NKCC2) were not altered.

Hypertension and Hypokalemia-Induced Activity of NCC

On the basis of the recent reports demonstrating that low plasma potassium levels lead to the phosphorylation of NCC,[20] we hypothesized that hypokalemia led to the activation of NCC in the knockout kidneys, and that NCC upregulation may be reversed by regulating the potassium levels. To test this hypothesis, we induced the elevation of plasma potassium levels in the knockout mice by chronically administering ENaC inhibitor, amiloride, or by applying 8% KCl diet (Figure 5A). As expected, the levels of T53-phosphorylated NCC in the knockout mouse kidneys decreased to the levels determined in the WT kidneys, whereas the levels of cleaved ENaCα were considerably increased after these treatments (Figure 5B and 5C; Figure S7), which was most likely affected by the plasma potassium level-induced aldosterone upregulation.

The effects of hypokalemia-induced NCC activation on BP were analyzed by increasing plasma potassium levels in the knockout mice. We showed that the chronic administration of amiloride (Figure 5D; Figure S8A) and 8% KCl diet (Figure 5E; Figure S8B) led to a decrease in the MBP values in the knockout mice to the levels determined in the WT mice.

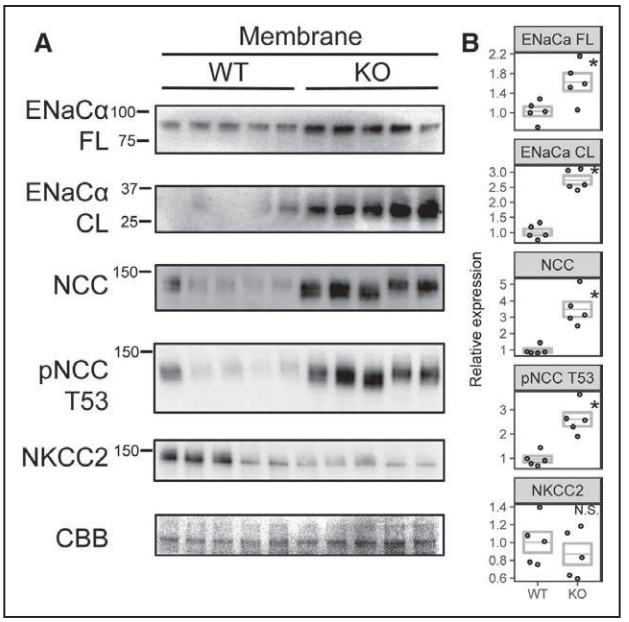

Figure 4. Upregulation of cleaved epithelial sodium channel-α (ENaCα) and phosphorylated NCC in the kidney of kidney-specific *Hsd11b2* knockout (KO) mice. **A**, The same amount of membrane fractions of kidney lysates were analyzed by Western blotting (WB). **B**, The expression levels, which were calculated from the intensities of WB band corrected by those of coomassie brilliant blue (CBB) staining (n=5 for each genotype), are shown as mean±SEM and statistically analyzed by Welch *t* test between the genotypes. **P*<0.05, compared with the control. CL indicates cleaved form; FL, full length; N.S., not significant; and WT, wild-type.

In addition, the MBP values in the knockout mice were significantly decreased, to the level of the WT mice, after the chronic administration of the NCC inhibitor, hydrochlorothiazide (Figure 5F; Figure S8C).

MR Dependency of the Hypertension

Finally, we aimed to examine whether MR is responsible for developing hypertension in the knockout mice. We subcutaneously administered slow-release pellets containing MR antagonist spironolactone to the knockout mice and measured their BPs. As a control, we administered MRA to the WT mice and measured their BP levels as well. We observed that MBP values significantly decreased after the administration of spironolactone in the knockout mice (Figure 6A), although MRA did not affect MBP values in the WT mice (Figure 6B). By chronic MRA treatment for 7 days, the expression level of cleaved ENaCα was significantly decreased in membrane fractions of the knockout kidney to the level of the WT kidney and NCC phosphorylation was partially but significantly decreased from its baseline (Figure 6C). Interestingly, renal NKCC2 was also decreased by chronic MRA treatment in membrane fractions of the knockout kidney (Figure 6C), in contrast to the no effects of amiloride or 8% KCl diet on the levels of renal NKCC2 (Figure 5B and 5C).

Discussion

Our analyses suggested that the complete kidney-specific deletion of *Hsd11b2* gene was achieved in the kidneys of

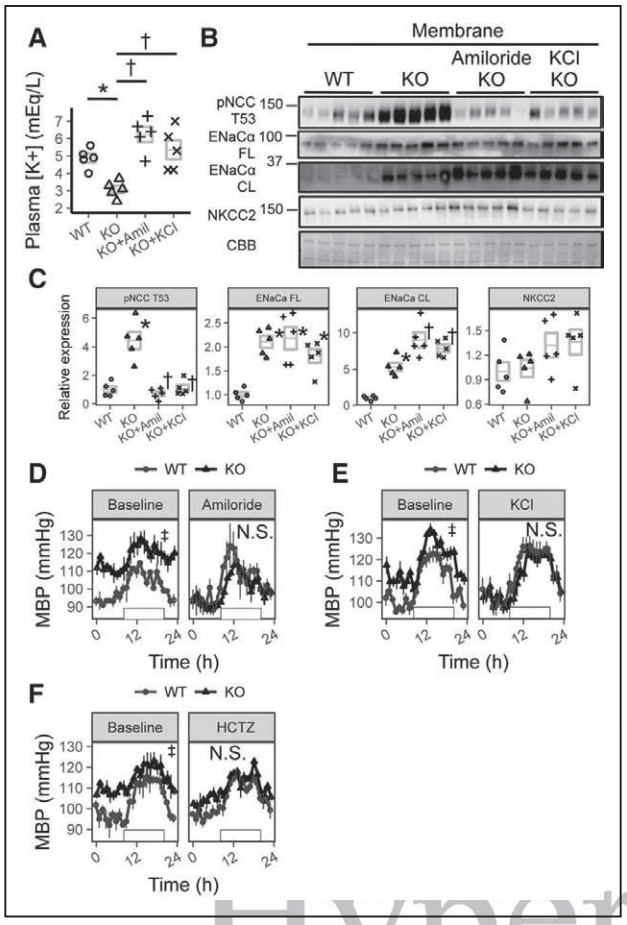

Figure 5. Effects of renal NCC activation on blood pressure levels in the kidney-specific *Hsd11b2* knockout (KO) mice. **A**, Plasma potassium concentrations of *Hsd11b2*$^{Ksp-/-}$ mice after treatment, amiloride (KO+Amil) or KCl diet (KO+KCl) and those of the control groups (wild-type [WT], KO) are shown (n=5 for each group). **B**, Results of Western blotting for transporter proteins in membrane fractions of kidney lysates and CBB staining; each WB band was quantified and corrected by the CBB intensity in the lane (**C**). BP recordings at baseline were obtained for 72 h, before the initiation of any treatment, and lasted for 10 d. The 3-h MBP in amiloride (**D**), 8% KCl diet (**E**), and hydrochlorothiazide (HCTZ, **F**) treated animals (n=3 in each group) are presented. Data are shown as mean±SEM and statistically analyzed by 1-way ANOVA and post hoc Bonferroni-Holm test (**A**, **C**) or 2-way ANOVA and post hoc Bonferroni–Holm test to assess the effects of the variables; genotype, and treatment. **P*<0.05 compared with the WT control; †*P*<0.05, compared with KO; ‡*P*<0.05, compared with WT in the same period. N.S. indicates not significant.

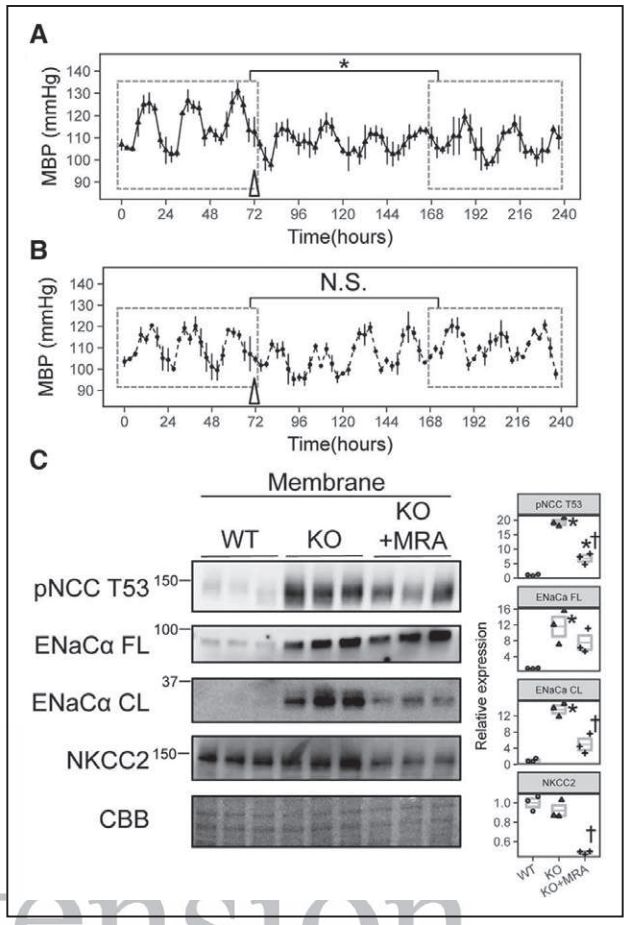

Figure 6. Effects of mineralocorticoid receptor (MR) antagonist spironolactone on the blood pressure levels of kidney-specific *Hsd11b2* knockout (KO) mice. After BP recording at baseline in *Hsd11b2*$^{Ksp-/-}$ mice (KO, **A**) and the wild-type (WT) controls (**B**) during days 1 to 3, a pellet containing spironolactone (MRA) was implanted to each group of mice (n=3) and blood pressure (BP) recording was continued until day 10. Averaged 3-h mean BP (MBP) are shown. Two-way ANOVA with post hoc Bonferroni–Holm test was performed to assess the effects of the valuables; genotype, and MRA treatment. **P*<0.05, compared with the baseline values. **C**, Renal membrane fractions of MRA-treated KO mice and the control groups (WT, KO) were analyzed by Western blotting for sodium transporter proteins; each band was quantified and the result was corrected by the intensity of CBB staining. These data were statistically analyzed by 1-way ANOVA with post hoc Bonferroni–Holm test. All data were shown as mean±SEM. **P*<0.05, compared with WT; †*P*<0.05, compared with KO. CL indicates cleaved form; and FL, full length.

Hsd11b2$^{Ksp-/-}$ mice. The results showing that the aldosterone levels and renin activity in plasma were lower, whereas the concentration of corticosterone was not altered in the knockout mice, in comparison with those in the WT mice, suggested that MBP elevation in the knockout mice can be attributed to sodium retention induced by kidney-specific induction of corticosterone levels, which leads to the suppression of circulating renin–angiotensin–aldosterone system. In addition, the obtained results were consistent with our hypothesis that HSD11B2 deficiency in the kidney is sufficient for the development of hypertension and other AME-like phenotype.

Recently, the mechanism of salt-sensitive hypertension has been vigorously discussed, which was initiated by the criticism of Guyton proposal. The critics highlighted the lack of normotensive subjects as controls for salt-sensitive hypertensive subjects, which showed a greater salt load–induced elevation of BP, primarily caused by fluid retention and followed by an increase in the total peripheral vascular resistance, in comparison with that in the nonsalt-sensitive hypertensive subjects.[8] Morris et al[8] reported the results of a study including human normotensive subjects. In the study, BP was elevated immediately after the salt loading in salt-sensitive subjects and the total peripheral vascular resistance decreased less, before an increase in the cumulative sodium balance was greater, in comparison with those in the salt-resistant subjects. The authors thus proposed that

vasodilatation dysfunction precedes the initiation of salt-sensitive hypertension.

In contrast, Hall *et al*[3] pointed out the lack of evidence demonstrating a decisive contribution of the abnormal nonrenal vascular resistance to the initiation of salt-sensitive hypertension. It was suggested that abnormal renal vascular resistance may have a role in the impaired renal sodium handling, and several studies investigating hypertension by kidney-specific manipulations were cited.[3] However, kidney-specific gene manipulations were not used to confirm the importance of gene mutations found in Mendelian hypertensive disorders for the development of renal dysfunction and systemic hypertension. Therefore, the possible nonrenal effects of these gene mutations should be considered as well.

Our aim was to determine whether kidney-specific gene mutations found in patients with AME syndrome can cause renal dysfunction leading to salt-dependent hypertension. Considering the increased activity of ENaC in the knockout mouse kidneys in comparison with the WT kidneys and the normalization of their BP after the amiloride treatment, we suggest that ENaC activation contributes to the development of salt-dependent hypertension in the kidney-specific *Hsd11b2* knockout mice. Furthermore, we determined that the activation of MR induced by the increased levels of corticosterone in the kidneys affects BP elevation in the knockout mice, and the application of MR antagonist showed that salt-sensitive hypertension can be attributed to MR activation, in aldosterone/corticosterone-dependent and aldosterone/corticosterone-independent fashion.[21,22] Designed in a more organ-specific manner than the previous studies, the results obtained here indicate the significance of renal dysfunction in explaining the mechanism underlying the development of Mendelian salt-dependent hypertension, although we did not evaluate either the cumulative sodium levels balance or hemodynamics such as pressure-natriuresis. Relative to this issue, total-body sodium storage, a large amount of which is sequestered in the skin, was reported to have a role in BP regulation.[23] Further studies are needed to clarify where either "sodium retention" or the total body sodium storage is essential for the development of sodium-dependent hypertension of our kidney-specific *Hsd11b2* knockout mice.

Chronic amiloride administration and high-KCl diet allowed the elucidation of the role of hypokalemia-induced renal NCC activation on the development of hypertension during renal HSD11B2 deficiency. These results suggest that ENaC and NCC, but not NKCC2, are activated in the knockout mouse kidneys, indicating that hypokalemia-induced NCC activation augments BP elevation induced by renal ENaC activation. These data support a universal significance of NCC activation by potassium-deficient diet-induced hypokalemia, as previously reported.[20] The antihypertensive effect of potassium was shown to be associated with the DOCA salt–treated hypokalemic rats[24] and here with the kidney-specific *Hsd11b2* mice as well. Furthermore, several studies[25–28] reported a consistent decrease in BP achieved by potassium supplementation in hypertensive patients and animals with normokalemia, particularly in patients of salt-sensitive hypertension with low plasma renin activity. In contrast to the effectiveness of potassium supplementation in "low-renin" hypertension, Veiras et al[29] recently reported that the BP elevation in chronic angiotensin II–infused rats, which were normokalemic, was not affected by KCl supplementation, suggesting that the anti-hypertensive effect of potassium supplementation is less pronounced in "high-renin" hypertension. Besides the natriuretic effect of potassium supplementation, the elevation of extracellular potassium levels was shown to lead to vasodilatation through the activation of barium-sensitive potassium channels in vascular smooth muscle cells.[30] Given high-salt-induced decrease in potassium-induced vasodilatation,[31] however, the antihypertensive effect of potassium supplementation in our animal model of salt-dependent hypertension can be attributed to natriuresis. These results strengthened the hypothesis that impaired renal function rather than vascular dysfunction contributed to the development of salt-dependent hypertension induced by kidney-specific *Hsd11b2* gene deletion. Taken together, potassium supplementation may have pronounced antihypertensive effects, especially in some hypertensive disorders accompanied with hypokalemia or with low plasma renin activity, such as AME syndrome and aldosterone breakthrough.[32]

Although brain-specific *Hsd11b2* knockout mice were hypertensive only when fed with extremely high-salt diet,[15] our kidney-specific *Hsd11b2* knockout mice, which were fed with normal-salt diet, developed definite hypertension, which was ameliorated by the administration of low-salt diet and diuretics, such as amiloride and hydrochlorothiazide. Predisposition to vasoconstriction was suggested as the mechanism underlying the development of hypertension in global and brain-specific *Hsd11b2* knockout mice by the experiments using α1-receptor agonist phenylephrine and its blocker prazosin; implying the contribution of impaired baroreflex and increased production of catecholamines, respectively.[15,16] In contrast, plasma levels of catecholamines were normal in a patient with AME syndrome.[17] The discrepancy between global knockout mice and patients with AME syndrome led us to a plausible hypothesis that, probably because of species difference in salt susceptibility of SNS activity, the phenotype of patients with AME syndrome is different from that of global knockout mice with the increased SNS activity, rather similar to that of the kidney-specific knockout mice with the intact baroreflex which was suggested by lower heart rate than the WT control mice in our study. Thus, the natriuretic and the BP-lowering effects of amiloride in kidney-specific knockout mice, which also appears in patients with AME syndrome, were supposed to be absent in global knockout mice with the increased SNS activity.

Therefore, we would like to assert that kidney-specific knockout mice is a better rodent model than global knockout mice for examining the mechanism of salt-dependent hypertension in patients with AME syndrome. On the basis of the previous finding indicating that salt loading-induced SNS overactivity could be corrected by potassium supplementation following natriuresis[33,34]; moreover, there are some possibilities that neural mechanism is secondary to salt retention in global knockout mice.[35] Further study is needed to

evaluate the mechanism of hypertension in terms of organ network in *Hsd11b2* knockout animals and in patients with AME syndrome.

Perspectives

Using the kidney-specific *Hsd11b2* knockout animal model, we revealed hypertension and hypokalemia, which are the expected phenotype of AME syndrome, in these animals. We demonstrated for the first time that kidney-specific HSD11B2 deficiency is sufficient for the development of the phenotype and that renal dysfunction is sufficient for BP increase in a model of Mendelian hypertension. Furthermore, we demonstrated that the activities of MR and ENaC in the kidney have a primary role in the development of the phenotype in the animal model of AME syndrome. Of note, NCC activity was shown to have a secondary but decisive role on developing hypertension in this animal model. The results of this study, therefore, suggest the importance of renal dysfunction in the pathology of a Mendelian hypertensive disorder, AME syndrome.

Sources of Funding

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Disclosures

None.

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Novelty and Significance

What Is New?

- Kidney-specific *Hsd11b2* knockout mice exhibited apparent mineralocorticoid excess–like phenotype including salt-dependent hypertension and hypokalemia with normal salt intake.
- Mineralocorticoid receptor (MR)–epithelial sodium channel (ENaC)–hypokalemia–Na^+-Cl^- cotransporter (NCC) pathway plays an important role in developing hypertension of kidney-specific *Hsd11b2* knockout mice.

What Is Relevant?

- Renal HSD11B2 deficiency is essential for developing the phenotype of apparent mineralocorticoid excess syndrome.
- Not only MR and ENaC but also hypokalemia and NCC are the therapeutic targets in the disease state of 11β-HSD2 deficiency.

Summary

This study suggests that 11β-HSD2 deficiency in the kidney causes salt-dependent hypertension through MR–ENaC–Hypokalemia–NCC pathway, thereby supporting the clinical importance of renal dysfunction in the pathology of Mendelian hypertensive disorders.

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Renal Dysfunction Induced by Kidney-Specific Gene Deletion of *Hsd11b2* as a Primary Cause of Salt-Dependent Hypertension

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ONLINE SUPPLEMENT

Renal dysfunction induced by kidney-specific gene deletion of *Hsd11b2* as a primary cause of salt-dependent hypertension

Running title:

Ueda et al, Hypertension with Kidney-specific Hsd11b2 KO

Authors:

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Expanded Materials and Methods

Generation of Kidney-specific Knockout Animals

Hsd11b2 deletion in renal tubular epithelial cells was achieved by mating C57BL/6-background transgenic mice expressing Cre-recombinase (Cre) under the control of Ksp-cadherin promoter (purchased from The Jackson Laboratory, USA), with the C57BL/6-background mice harboring *Hsd11b2*-floxed alleles, [1] which resulted in C57BL/6-background $Hsd11b2^{Ksp-/-}$ mice. During the process of generating $Hsd11b2^{Ksp-/-}$ mice, we also produced Ksp-Cre positive or negative mice with wild-type (non-floxed) *Hsd11b2* gene, which were C57BL/6 background as well as the founders. All animals were age-matched in each experiment.

Blood Analyses

Animal blood samples were obtained from inferior vena cava (IVC) at the sacrifice using heparin-coated syringes and immediately analyzed electrolyte plasma concentrations by using i-STAT System 6+ Cartridge (Abbott, USA). Blood samples were centrifuged at 2300 ×g for 10 min, and the supernatants (plasma samples) were collected and stored at -80°C. Plasma aldosterone levels and renin activity were measured by radioimmunoassay, while plasma corticosterone concentrations were measured by using ELISA.

Urine Analyses

Before placing a mouse in a metabolic cage, the urinary bladder was emptied by abdominal massage. Urine was collected during 24 h (starting at 12 PM) from mice placed in metabolic cages. Urinary corticosterone and 11-dehydrocorticosterone contents were determined using Liquid chromatography-mass spectrometry (LC-MS).

Blood Pressure Measurement Using Telemetry System

Arterial BP in conscious mice was directly monitored by catheterization of left carotid artery with radiotelemetry, using a PA-C10 transmitter, RPC-1 receiver, APR-1 ambient pressure monitor, and a Data-Quest-ART-Silver 4.2 acquisition system (Data Sciences International, USA). The transmitter was placed subcutaneously. After the recovery for 1 week from the surgical procedure, systolic/diastolic BP and heart rate were measured continuously, for 10 s every 15 min, and an hourly or 3 hours mean BP was calculated in the usual manner by averaging sequential systolic/diastolic BP

values. BP recording was initiated at 12 PM on day 1. BP values obtained for the first 3 days at baseline were compared to those for the last 3 days with the treatment in each experiment.

Blood Pressure Measurement using Tail-cuff method

Systolic blood pressure (SBP) of conscious animals was measured at light phase by using tail-cuff method; BP-98A (Softron). SBP was averaged as the average of 5 measurements for each mouse. SBP values of Ksp-Cre positive or negative mice were obtained by turns to minimize the effect of circadian rhythm on SBP.

Protein Sample Preparation and Western Blotting

Total proteins were obtained by using TNE buffer containing 10 mM Tris-HCl (pH 7.8), 1% NP-40, 0.15 M NaCl, and 1 mM EDTA. Membrane fractions were obtained with Minute^{TM} Plasma Membrane Protein Isolation Kit (Invent Biotechnologies, Inc., USA) and resuspended in the lysis buffer containing 40 mM Tris (pH 7.4), 260 mM sucrose, 1% Triton X-100, and cOmplete Protease Inhibitor (Roche, Switzerland). In order to detect phospholyrated NCC, phosphatase inhibitors (10 mM NaF, 1 mM NaP2O7, and 1 mM NaVO4) were added to all buffers. Protein concentrations were determined by BCA protein assay kit (Thermo Fisher Scientific, USA). Equal volumes of lysates were mixed with 2× Laemmli Sample Buffer, and the samples were incubated at 20°C for 30 min. Equal amounts of total proteins were loaded in each well of SDS-PAGE. The protein-transferred membranes were incubated with ECL Prime Blocking reagent for 1 h at room temperature, and further incubated with one of the primary antibodies at 4°C overnight. Following this, the membranes were incubated with peroxidase-conjugated secondary antibodies for 30 min at room temperature, and the obtained results were visualized by using ImmunoStar LD (Wako Chemicals, Japan). Bio-Safe Coomasie G-250 stain (Bio-rad) was used for CBB staining. Densitometry was done by using ImageJ 1.50i. The intensity of WB band was corrected by that of CBB staining to calculate the expression level of each protein.

Antibodies

In addition to the listed antibodies in the main Methods, primary antibodies used in this study were as follows: anti-NCC (1/1000 for IHC, AB3553, Millipore); anti-HSD11B2 (1:1000 for WB; AB1296, Millipore); anti-HSD11B1 (1:1000, for WB; ab39364, Abcam); anti-AQP2 (1/100 for IHC, sc-9882, Santa Cruz Biotechnology);

anti-NPM (1/3000 for WB, B0556, Sigma-Aldrich); anti-α-Tubulin (1/3000 for WB, 017-25031, Wako Chemicals); anti-pan-cadherin (1/1000 for WB, C3678, Sigma).

Acute Amiloride Test

At day 1 (12 PM), vehicle solution (2% dimethyl sulfoxide (DMSO) in distilled water) was intraperitoneally injected into WT and KO mice (n=4 in each group), and they were placed in a metabolic cage. Urine samples were collected at 3 PM on the same day. At day 2 (12 PM), amiloride (5 mg/KgBW) was intraperitoneally injected and the urine samples were collected at 3 PM. The samples were centrifuged at 2000 ×g for 10 min at 4°C immediately after the collection, and the supernatants were used in the following analyses. Blood samples were collected from IVC after the sacrifice.

Immunohistochemical Analysis

Mouse kidneys were fixed with 4% paraformaldehyde (PFA) immediately following the animal sacrifice, and they were rotated overnight at 4°C. Cryosections of the fixed mouse kidneys were incubated with one of the primary antibodies overnight, which was followed by an incubation with Alexa488- or Alexa555-conjugated secondary antibodies (Jackson ImmunoResearch, USA) for 1 h at 4°C and DAPI (1/5000) at room temperature for 5 min. The images were obtained using DM14000B microscope and LAS AF software (Leica, Germany).

Gene Expression Analyses

Total RNA was obtained from tissue homogenates by using RNeasy Mini Kit (Qiagen, Germany), while cDNA was synthesized using ReverTra Ace (Toyobo, Japan). Quantitative realtime PCR analysis was performed using the StepOne Plus detection system (Applied Biosystems, USA). SYBR primers used in this study are shown in *Supplemental Table*.

Supplemental Table. SYBR primers.

Gene	Forword	Reverse
Hsd11b2	GGCTGCTTCAAGACAGATGC	TGGGCTAAGGTCAGGCAATG
Hsd11b1	GGAGCCGCACTTATCTGAAG	GTAGTAGGCCAGGGAGAGCA
Cdh16	CTTCAAGGGCATGGTCTAGG	CCACCTCAGCCTTTACCAGA
Pecam1	CTTTTCGAGGTGGTGCTGAT	CCTCCAGGCTGAGGAAAACT
NeuN	CGCTGAACCCGTTGAAAAGC	TTCCCCCTGGTCCTTCTGAT

Supplemental Reference

1. Jiang L, Yang S, Yin H, Fan X, Wang S, Yao B, Pozzi A, Chen X, Harris RC, Zhang M-Z. Epithelial-specific deletion of 11β-HSD2 hinders Apcmin/+ mouse tumorigenesis. *Mol Cancer Res*. 2013;11(9):1040-1050.

Supplemental Figures

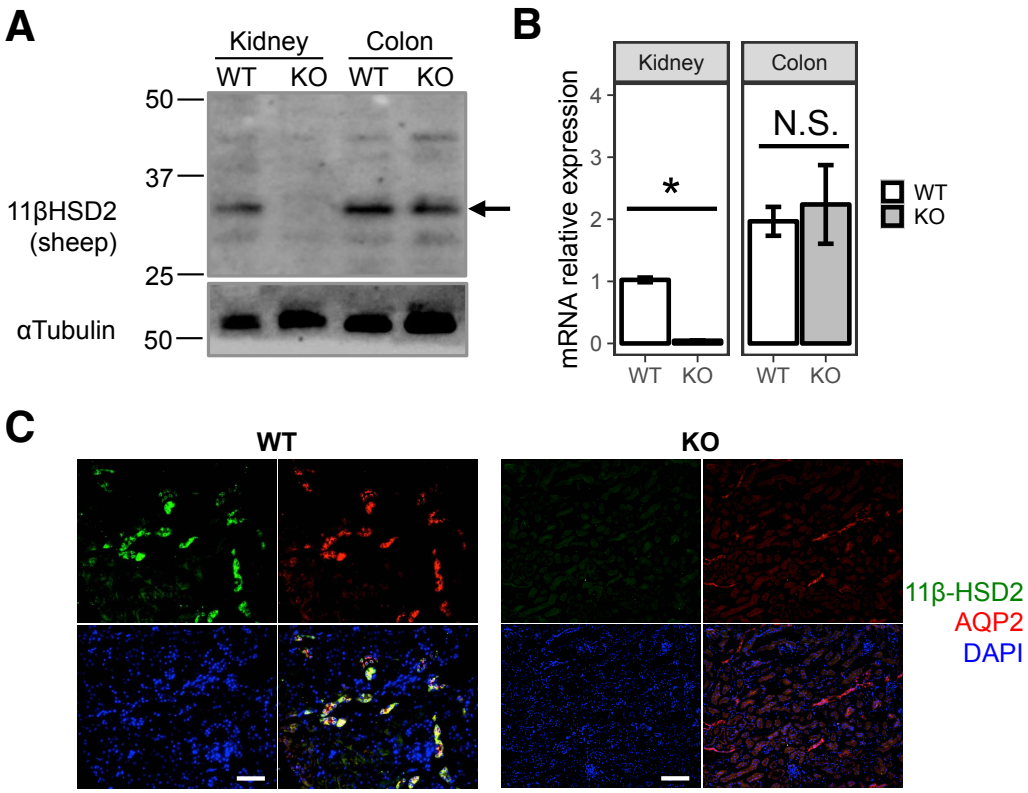

Figure S1. Kidney-specific deletion of *Hsd11b2* gene.
Protein (**A**) and mRNA (**B**) HSD11B2 levels were determined in *Hsd11b2*$^{KSP-/-}$ mice (n=3) and the littermate controls (n=3). Kidney-specific gene deletion was confirmed by measuring HSD11B2 expression levels in colon. Immunofluorescence analysis (**C**) showed that *Hsd11b2* was deleted in AQP2-expressing renal tubular principal cells. Bar represents 50 μm. Data are shown as mean ± SEM. One-way ANOVA with post-hoc Bonferroni-Holm test was used for the statistical analysis. *P<0.05, compared with the control.

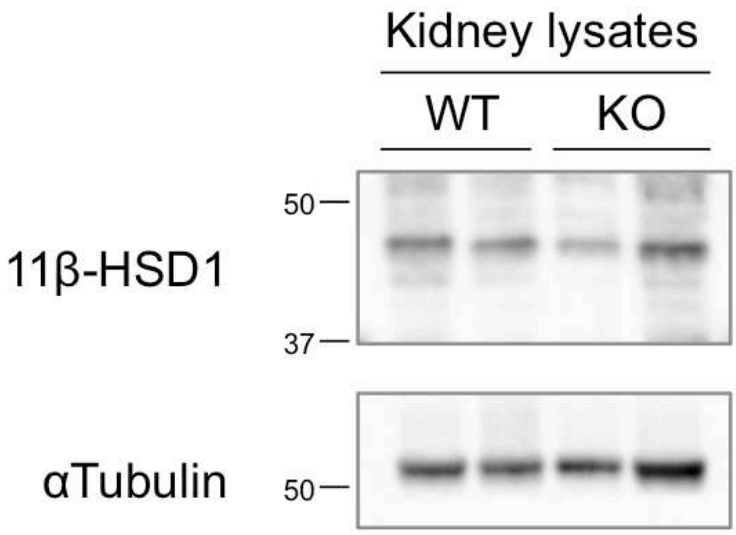

Figure S2. Expression level of 11β-hydroxidehydrogenase type 1 in the kidneys of kidney-specific *Hsd11b2* knockout mice.

Western blot analysis of showed that the renal expression levels of HSD11B1 were not affected by kidney-specific deletion of *Hsd11b2* gene.

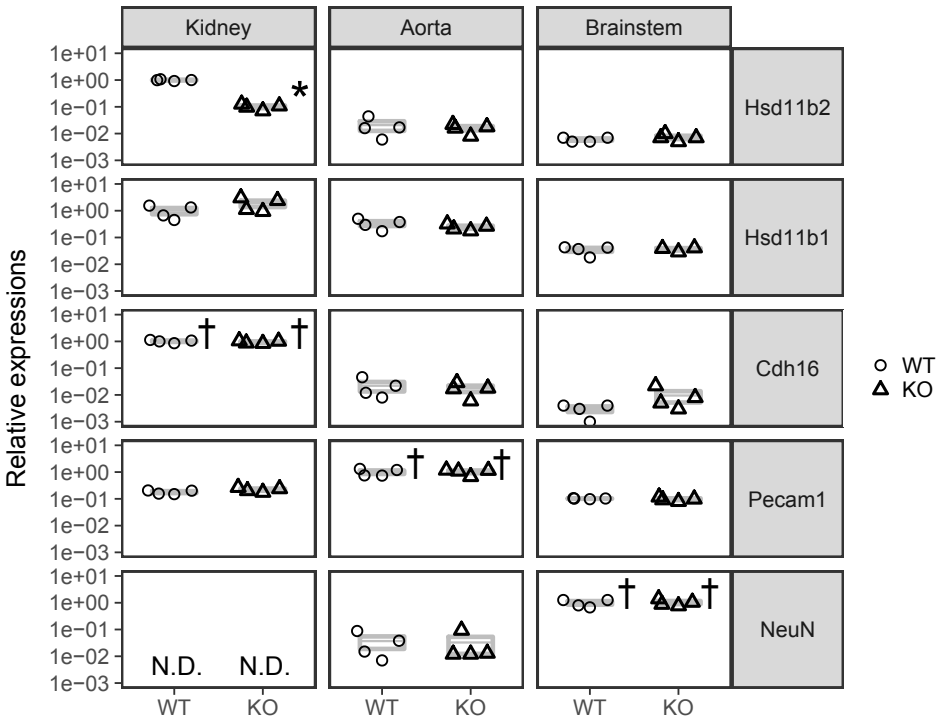

Figure S3. Transcriptional expression level of *Hsd11b2* and *Hsd11b1* gene in *Hsd11b2*-expressing tissues of kidney-specific *Hsd11b2* knockout mice. RNA was obtained from kidney, aorta and brainstem of Hsd11b2$^{Ksp-/-}$ mice (KO, n=4) and the littermate controls (WT, n=4). The transcriptional expression level of HSD11B2 and HSD11B1 were statistically analyzed along with tissue markers between the genotypes in each tissue by using one-way ANOVA with Bonferroni-Holm test. Data are presented as mean ± SEM. N.D., not detected. *P<0.05, compared to the WT tissue; †P<0.05, compared to other tissue of each genotype.

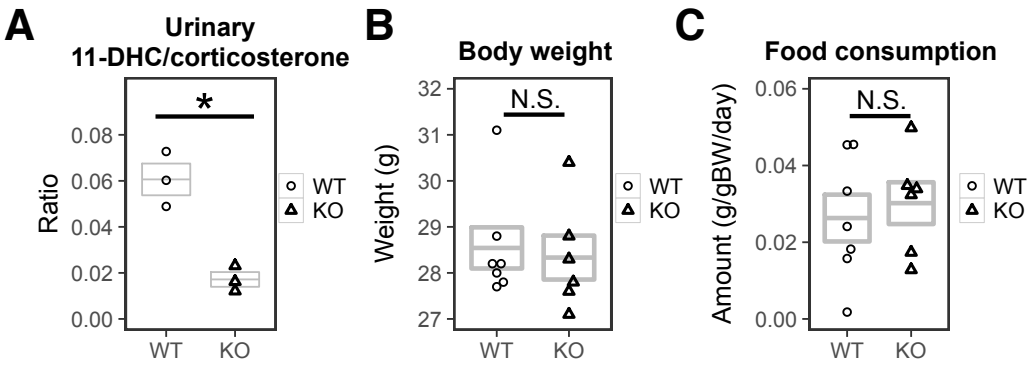

Figure S4. 11-Dehydrocorticosterone/corticosterone ratio in urine samples, body weight, and daily food intake of the experimental animals.

The ratio of 11-dehydrocorticosterone (11-DHC) and corticosterone (**A**, n=3 for each genotype), body weight (**B**, WT; n=7: KO; n=6), daily food intake (**C**, WT; n=7: KO; n=6) of kidney-specific *Hsd11b2* knockout mice and controls. Welch's t-test was used for the statistical analyses. *P<0.05, compared with the control.

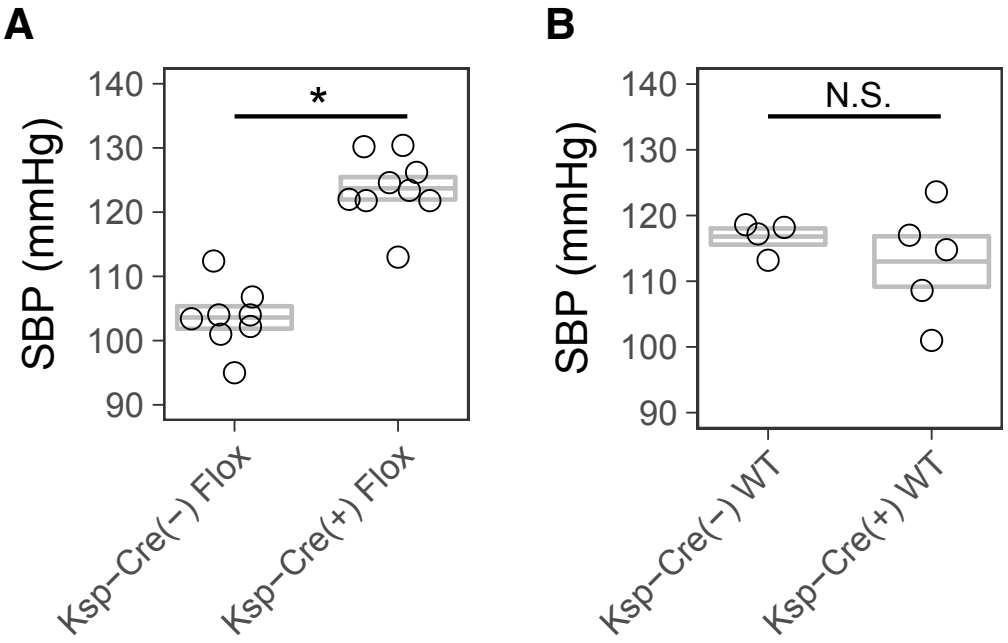

Figure S5. Negated effect of *Ksp-Cre* transgene on elevating systolic BP.
A, Systolic BP (SBP) values were evaluated by tail-cuff method in the WT mice (Ksp-Cre(-) Flox; n=8), the KO mice (Ksp-Cre(+) Flox; n=9). **B**, SBP values were also obtained from mice harboring wild-type *Hsd11b2* gene without *Ksp-Cre* transgene (Ksp-Cre(-) WT; n=4) and those with *Ksp-Cre* transgene (**B**, Ksp-Cre(+) WT; n=5). Welch's t-test was used for the statistical analyses between the genotypes in each experiment. Data are shown as mean ± SEM. *P<0.05, compared with the control.

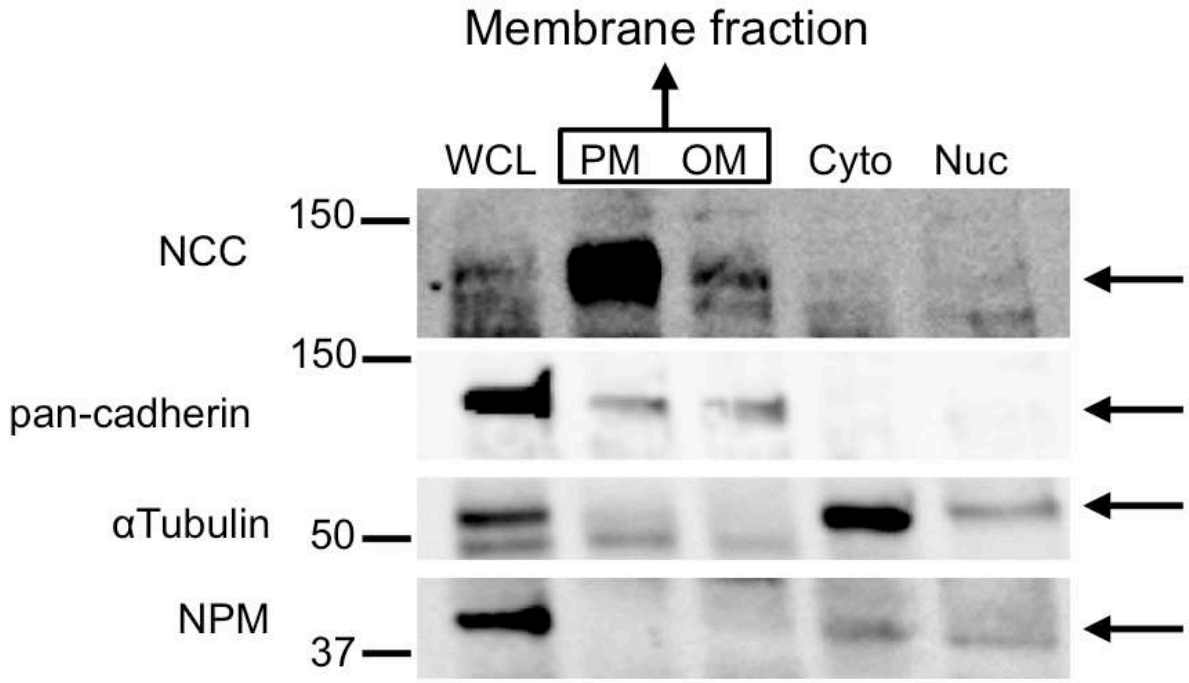

Figure S6. Fractionation of membrane proteins from kidney lysates. Whole-cell kidney lysates (WCL) were separated into four fractions: PM, plasma membrane; OM, organelle membrane; Cyto, cytoplasm; Nuc, nucleus. The fraction obtained before separating PM and OM represents a total membrane fraction, and it was used as such in this study. NCC and pan-cadherin were shown to be enriched in membrane fraction, PM, and/or OM, although cytoplasmic (α-tubulin) or nuclear protein (NPM, nucleophosmin) were not.

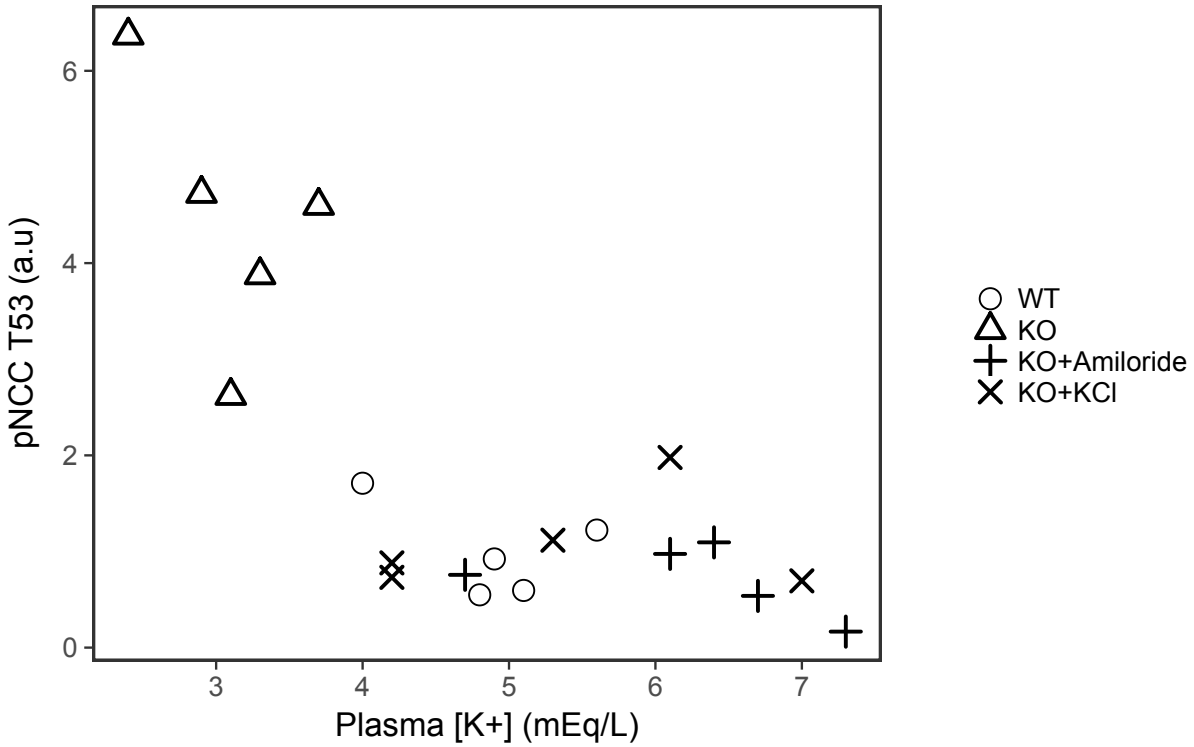

Figure S7. Hypokalemia and NCC phosphorylation level in kidney-specific *Hsd11b2* knockout mice.

Expression levels of renal NCC phosphorylation and plasma potassium concentration in kidney-specific *Hsd11b2* knockout mice (KO) and the littermate controls (WT) are shown, including the results of chronic treatments of amiloride or high KCl diet. a.u.; arbitrary unit.

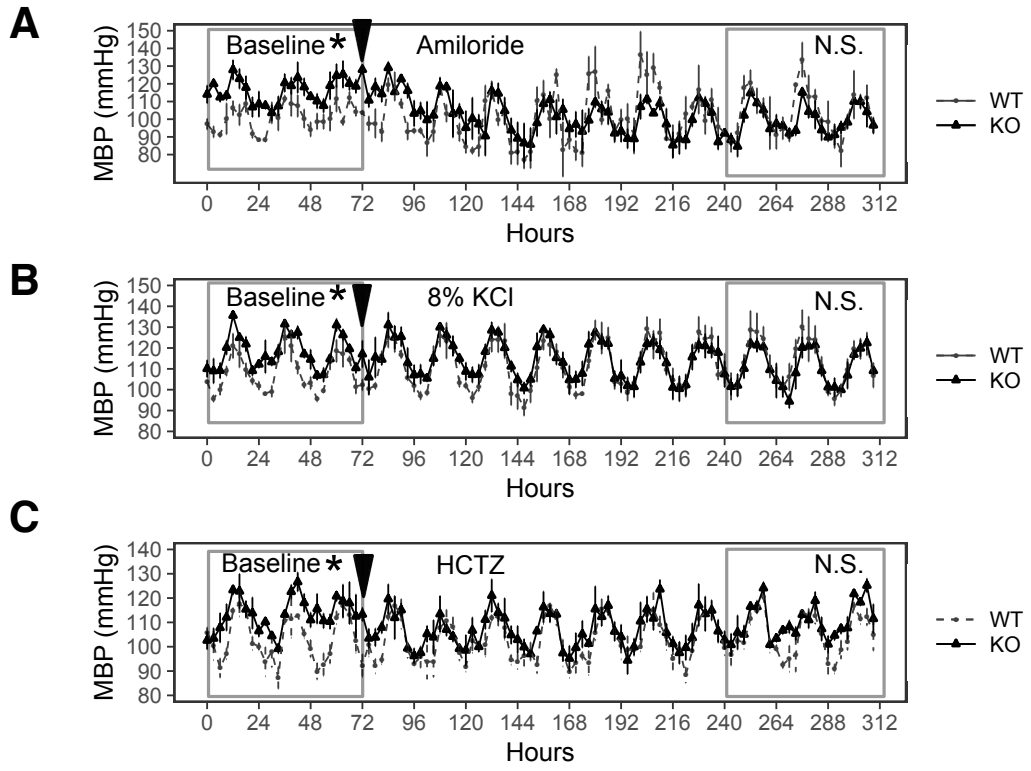

Figure S8. Continuous blood pressure (BP) measurements in kidney-specific *Hsd11b2* knockout mice and wild-type controls treated with amiloride or hydrochlorothiazide, or fed by 8% KCl diet.

BP was continuously measured for 72 h in *Hsd11b2*$^{Ksp-/-}$ mice and wild-type controls (n=3 in each group) (baseline measurements). Following this, chronic amiloride in drinking water (**A**), 8% KCl diet (**B**), or chronic hydrochlorothiazide in drinking water (**C**) were administered to the animals (arrowhead), and BP was measured for the additional 10 days. Hourly mean blood pressures were calculated and shown as mean ± SEM. Statistical analyses were performed by using two-way ANOVA with post-hoc Bonferroni-Holm test. *P<0.05, compared with baseline BP determined in the wild-type mice.